U.S. DIVISIONAL PATENT APPLICATION

OF

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FOR

UNITED STATES LETTERS PATENT

ON

AGENT FOR LOWERING PROLACTIN

Docket: 18810-81002
Sheets of Drawings: 1

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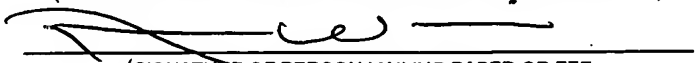

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Agent for Lowering Prolactin

The present invention relates to prolactin lowering drugs.

Extracts from Vitex agnus-castus (*agnus castus*, chaste tree) have been used for long in the medicine of natural remedies for treatment of the premenstrual syndrome. Shortly prior to menstruation, patients frequently complain of tenseness in the breasts, clinically accompanied by an elevated prolactin content.

Extracts from Vitex agnus-castus possess prolactin lowering properties which could furthermore be ascertained clinically and pharmacologically in the prior art. Attempts have been numerous in the prior art to characterize or even isolate those substances in Vitex agnus-castus that are responsible for an alleviation of the premenstrual sydrome.

Thus the dissertation by Daniel Berger entitled, "*Vitex agnus-castus: Unbedenklichkeit und Wirksamkeit beim prämenstruellen Syndrom, Wirkprinzipien and Wirkmechanismen eines neu entwickelten Extraktes*" [Vitex agnus-castus: Recognized safety und effectivity in the premenstrual syndrome, effective principles and mechanisms of a newly developed extract] at the Faculty of Philosophy and Natural Science of Basle University, of January 13, 1998 deals with a multiplicity of aspects brought into connection with Vitex agnus-castus.

This dissertation describes a number of different constituents which were taken into consideration for an explanation of the pharmacological properties of the drug.

Thus in Vitex agnus-castus the iridoid glycosides aucubin, agnuside and eurostoside are found both in the leaf drug and in the fruit drug.

Moreover the lipophilic flavonols casticin, penduletin, chrysosplenol D and the 3,6,7,4'-tetramethyl ether of 6-hydroxykaempferol could be isolated from the fruit.

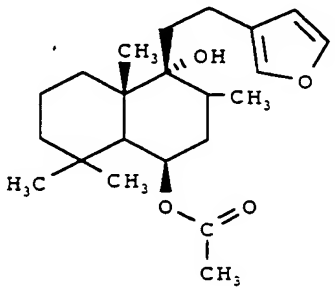
The prior art furthermore describes that progesterone, 17α-hydroxy-progesterone, testosterone and epitestosterone could be detected in the fruit of Vitex agnus-castus.

Apart from this, a total of about 73 different compounds can be found in the fruit of Vitex agnus-castus, above all monoterpenes such as α-pinene, sabinene, β-phellandrene and 4-terpineol, and sesquiterpenes such as β-caryophyllene, *allo*-aromadendrene, germacrene B, spathulenol and τ-cadinol.

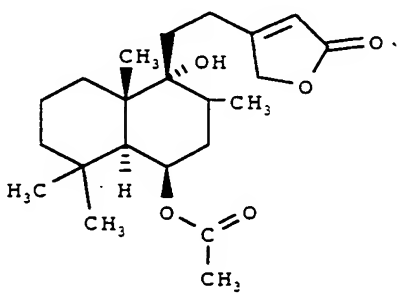
Besides the classes of substances already mentioned above, considerable amounts of fatty acids can moreover be found in the fruit of Vitex agnus-castus, as there are saturated, monounsaturated and polyunsaturated fatty acids. Thus, e.g., α-linolenic acid, oleic acid, stearic acid, palmitic acid, linoleic acid and adrenic acid were detected in the fruit.

Further examination of the essential oil from the fruit of Vitex agnus-castus also uncovered the presence of diterpenes. The above mentioned dissertation provides information that the following diterpenes were isolated from the fruit of Vitex agnus-castus:

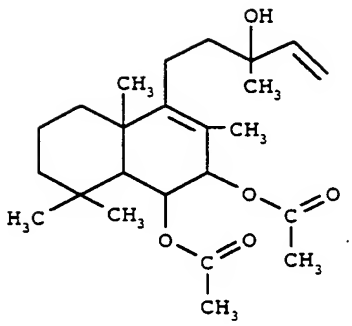
rotundifuran, vitexilactone and 6β,7β-diacetoxy-13-hydroxy-labda-8,14-diene, the structural formulae of which are represented hereinbelow:



Rotundifuran



Vitexilactone



6β,7β-Diacetoxy-13-hydroxy-labda-8,14-diene

In this prior art a multiplicity of testing processes were performed in order to find out about the effective mechanisms of extracts from Vitex agnus-castus, and whether a particular substance or a particular class of substances is suited for explaining the pharmacological effects of the full extract.

Thus, e.g., measurements were carried out on the various opioid receptors, on the benzodiazepin receptor, on the serotonin reuptake site, on the histamine-H_1 receptor and on the dopamine-D_2 receptor.

In order to verify the results of the receptor binding studies on the dopamine-D_2 receptor with fractions from Vitex agnus-castus and thereby find the actual active substances, experimentation was carried out in the above described prior art with the known constituents of Vitex agnus-castus (pure substances). These pure constituents were aucubin, casticin, homoorientin, linoleic acid, luteolin-7-glycoside, orientin and the diterpenes vitexin, rotundifuran, 6β,7β-diacetoxy-13-hydroxy-labda-8,14-diene.

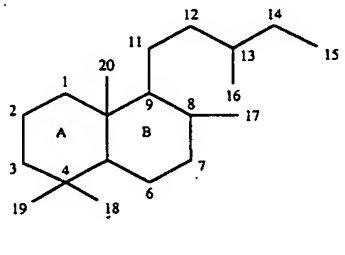
The dissertation does, however, explicitly state on page 154 in Chapter 2.3.4.5 that none of the tested substances had a sufficiently low IC_{50} value for being able to exaplain, as a single substance, the activity and thus the pharmacological effects of the full extract or of a lipophilic hexane fraction from Vitex agnus-castus.

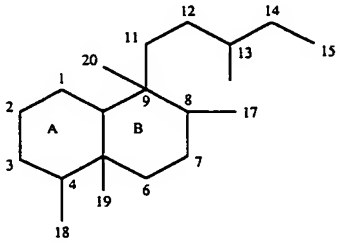
Starting out from this prior art, it was an object of the present invention to provide pure substances from the fruit of Vitex agnus-castus, whereby a drug for treating the premenstrual syndrome may be produced in pharmaceutical formulation.

This object is attained by a drug in accordance with claim 1 and by the novel substances in accordance with claim 12 and claim 14.

In the framework of the present invention it was surprisingly found that compounds from the class of bicyclic diterpenes have a prolactin lowering effect on cultivated pituitary cells from rats. It is highly likely that this mechanism can be transferred to humans.

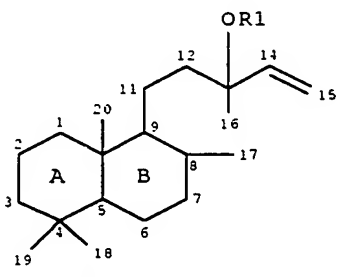
Herein the effective diterpenes may have a skeletal structure both of the labdane type and of the clerodane type:



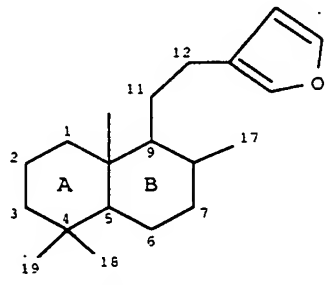


Labdane structure Clerodane structure

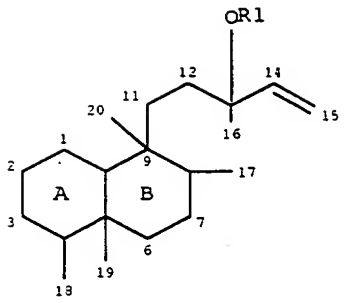
In particular it was found that a prolactin lowering effect on cultivated pituitary cells may be achieved with compounds in accordance with the following general formulae I to IV with, at the same time, low cytotoxicity:



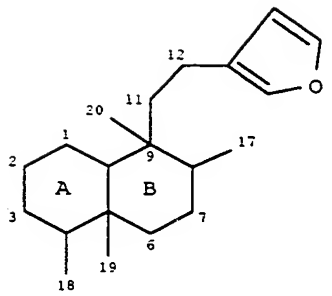
(I)



(II)



(III)



(IV)

with R1 = H, C1 to C3 alkyl or C1 to C3 acyl;

wherein the rings A and/or B in the case of general formulae (I) or (II) are optionally substituted in position 1, 2, 3, 4, 6, 7, 8 or 9 with at least one OX radical, with X = H, C_1 to C_3 alkyl or C_1 to C_3 acyl;

wherein the rings A and/or B in the case of general formulae (III) or (IV) are optionally substituted in position 1, 2, 3, 4, 6, 7, or 8 with at least one OX radical, with X = H, C_1 to C_3 alkyl or C_1 to C_3 acyl;

wherein optionally at least one carbon atom in positions 17, 18, 19 and 20 is substituted with an OX radical, with X = H, C_1 to C_3 alkyl or C_1 to C_3 acyl;

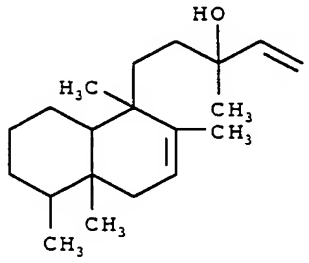
wherein optionally at least one CH_3 group in positions 17, 18, 19 and 20 is replaced by a COOH group;

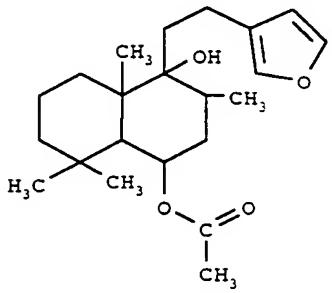
wherein optionally at least one of ring positions 1, 2, 3, 6, or 7 is a keto group; and

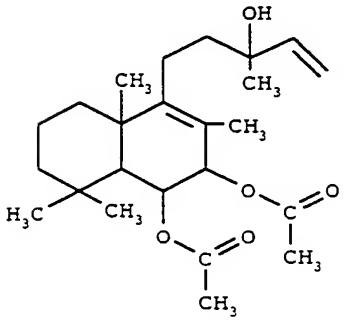
wherein optionally at least one double bond is present in ring positions 1, 2, 3, 6, 7, 8, 8(17) of formulae (I) and (III); and

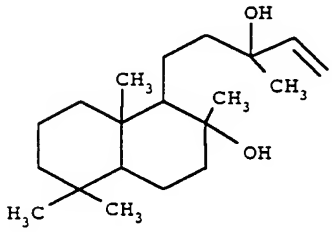
wherein optionally at least one double bond is present in ring positions 1, 2, 3, 4(18), 6, 7, 8, 8(17) of formulae (II) and (IV).

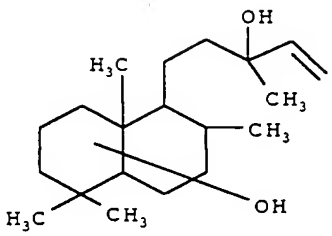
Moreover the following compounds are preferred embodiments of the present invention:

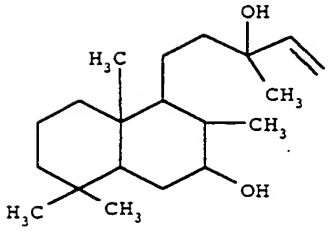


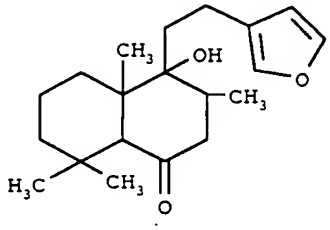


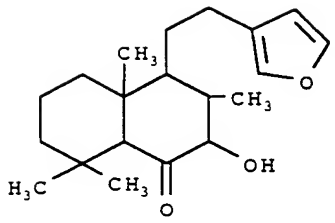


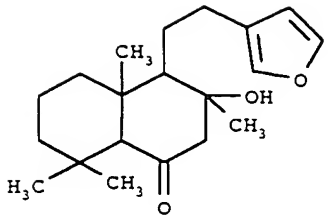


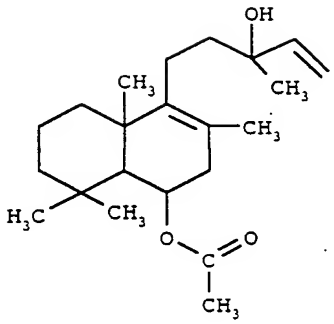












as well as

cleroda-Y,14-dien-13-ol, with Y = ring position 1, 2, 3, 4(18), 6, 7 or 8(17); and

cleroda-Y,Z,14-trien-13-ol, with Y or Z = ring position 1, 3, or 1, 4(18) or 1, 6 or 1, 7 or 1, 8(17) or ring position 2,4(18) or 2, 6 or 2, 7 or 2, 8(17) or ring position 4(18), 6 or 4(18), 7 or 4(18), 8(17) or ring position 6, 8(17).

It was possible to enrich and characterize the like compounds from an ethanolic-aqueous extract from fruit of Vitex agnus-castus by fractionated lipophilic extraction, and to determine their structures.

In particular extraction with highly lipophilic solvents such as medium-length chain hydrocarbons C_5-C_{10}, in particular with n-hexane, resulted in strong enrichment of the prolactin lowering effect. Moreover extraction from fruit of Vitex agnus-castus with supercritical carbon dioxide allowed for strong enrichment of the effective principle, which could be reduced to the compounds in accordance with general formulae I to IV.

All of the compounds in accordance with the invention, the structural formulae of which are represented above, exhibit inhibition of the released prolactin on lactotropic cells from rats' pituitary glands.

In separate studies on cytotoxicity it was found that all of the compounds isolated and characterized in accordance with the invention exhibited low cytotoxicity, a fact that renders them particularly attractive in terms of pharmaceutical formulation.

It was furthermore found that the named substances also bind to the human recombinant dopamine-D2 receptor.

Further advantages and features of the present invention result from the description of embodiments and from the drawing, wherein:

Fig. 1 shows the influence of bicyclic diterpenes on prolactin release from cultivated pituitary cells of rats.

An ethanolic extract from Fructus Agni casti is produced in a manner known per se by maceration or percolation with organic solvents or mixtures of organic solvents with water or with supercritical carbon dioxide.

To this end, preferably mixtures of ethanol with water in a ratio of 50:50 to 90:10 at a temperature from 20 to 60 degrees Celsius are used.

The extract obtained in this manner is distributed between two non-miscible phases having different polarities. Herein alkanes, halogenated hydrocarbons, ketones, esters are used as a lipophilic phase, and alcohols and water as a hydrophilic phase. Advantageously identical volumes of C5 to C7 alkanes and ethanol/water mixtures in a ratio of 1:2 to 1:10 are used.

The lipophilic phase contains the prolactin lowering activity and may be further purified with the aid of known methods, such as e.g. high-pressure liquid chromatography and preparative layer chromatography in a manner known per se.

From 1 kg of ground fruit of Vitex Agnus castus an extract is produced by percolation with 10 l of ethanol/water 6/4 (v/v). An inspissated extract produced therefrom and having a dry residue of 1.75 g is distributed

between 375 ml of 15% EtOH and 375 ml of n-hexane in the separating funnel, the n-hexane phase is withdrawn, and the aqueous phase is again extracted by shaking with n-hexane.

Following concentration under reduced pressure, the combined hexane phases give a residue of 300 mg.

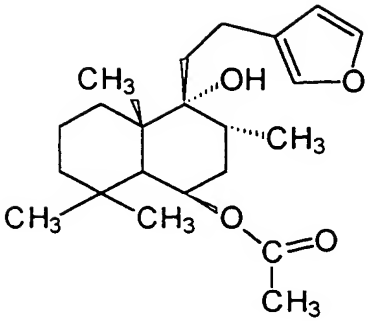
The residue thus obtained is further separated by means of high-pressure liquid chromatography. For this a column having the dimensions of 21.4 × 300 mm is used, with C-18 material having a particle size 8 µm as the stationary phase. Chromatography is carried out at a flow of 10 ml/min of a mixture of acetonitrile/water 60/40 as the solvent. Following charging of the sample, the acetonitrile content is linearly increased to 100% within 60 minutes.

All of the diterpenes elute in the volume between 350 and 450 ml. From 300 mg of hexane phase, approx. 38 mg of mixture of diterpenes are obtained. The diterpenes are suitably collected in the form of fractions.

Further purification of the mixture of diterpenes thus obtained is performed by means of preparative layer chromatography on silica gel layers having a layer thickness of 1 mm, with different flow agents in accordance with the description further below for the single substances. Detection is performed with anisic aldehyde reagent (DAB 10, 1997). The zones of the pure diterpenes on the thin-layer plate are eluted with the aid of chloroform/methanol and analyzed by means of coupled gas chromatography mass spectrometry.

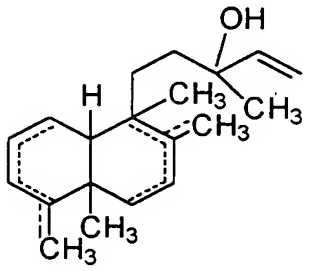
Preparation of 98-146 (designated by "146" in Fig. 1):

6ß-Acetoxy-9α-hydroxy-15,16-epoxy-13(16),14-labdadiene (rotundifuran)

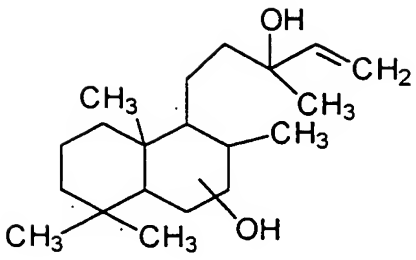


Flow agent: chloroform / methanol 95/5
Rf value: 0.75
Characteristic fragments of the underivatized substance in GC-MS:
m/z=362 [M]+ , 344, 302, 287, 284, 207, 150, 135, 95, 81.

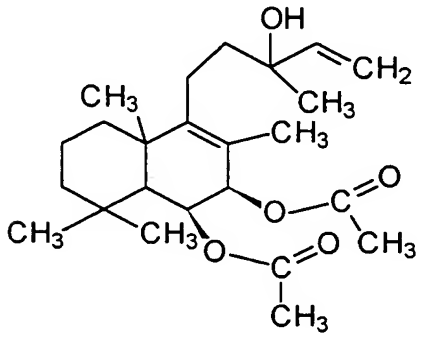
Preparation of 98-119 (designated by "119" in Fig 1.):
Cleroda-y,14,-dien-13-ol
Flow agent: chloroform / methanol 95/5
Rf value: 0.63
Characteristic fragments of the underivatized substance in GC-MS:
m/z=290 [M]+ , 272, 257, 243, 229, 191, 189,



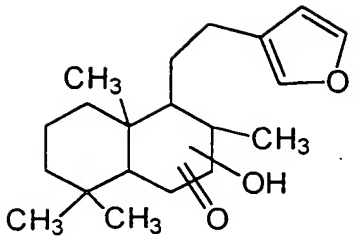
Preparation of 98-153 (not shown in Fig. 1):
x,13-Dihydroxy-14-labdene
Flow agent: chloroform / methanol 95/5
Rf value: 0.37
Characteristic fragments of the underivatized substance in GC-MS:
m/z=290 [M-H2O]+ , 275, 272, 257, 191, 177



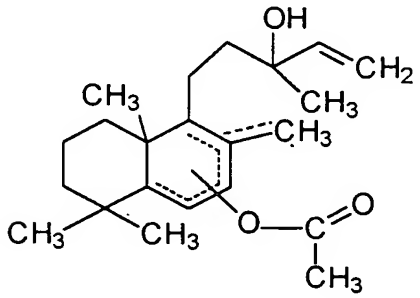
Preparation of 98-152 (not shown in Fig. 1):
6ß,7ß-Diacetoxy-13-hydroxy-labda-8,14-diene
Flow agent: chloroform / methanol 99/1
Flow distance 16 cm, developed 3×
Rf value: 0.5
Characteristic fragments of the underivatized substance in GC-MS:
m/z=346 [M-60]+ , 307, 304, 286, 247, 205, 187, 177, 135



Preparation of 98-166 (designated by "166" in Fig. 1):
x-Hydroxy-y-keto-15,16-epoxy-13(16),14-labdadiene
Flow agent: chloroform / n-hexane 90/10
Develop 3×, flow distance 16 cm
Rf value: 0.74
Characteristic fragments of the underivatized substance in GC-MS:
m/z=318 [M]+ , 300, 285, 193, 166, 95, 81



Preparation of 98-167 (designated by "167" in Fig. 1):
x-Acetoxy-13-hydroxy-labda-y,14-diene
Flow agent: chloroform / n-hexane 90/10
Develop 3×, flow distance 16 cm
Rf value: 0.55
Characteristic fragments of the underivatized substance in GC-MS:
m/z=330 [M-H2O]+ 288, 270, 255, 249, 189, 132, 119, 71



Influence on prolactin release:

Determination of prolactin release from cultivated pituitary cells of male rats was performed as described in Jarry et. al., Experimental and Clinical Endocrinology, Vol. 102, (1994) 448 - 454. The diterpenes were added to the cell cultures in ethanolic solution. The corresponding ethanol concentrations and dopamine were carried along as controls.

The mean value of the measured prolactin concentration of supernatants in cells incubated in unsupplemented medium is set to be equal 100%. The diterpenes significantly lower the release of prolactin. The results are represented in Fig 1. It shows lowering of prolactin release from cultivated

pituitary cells of rats through bicyclic diterpenes. As a control, medium, medium plus ethanol and 10^{-4} molar dopamine (=DA-4M) were carried along. The concentration is indicated in mg of diterpene per ml of medium.